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Apoptotic blebs from *Leishmania major*-infected macrophages as a new approach for cutaneous leishmaniasis vaccination

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Title:

Apoptotic blebs from *Leishmania major*-infected macrophages as a new approach for cutaneous leishmaniasis vaccination

Running title:

Apoptotic blebs and cutaneous leishmaniasis vaccination

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Abstract

We focused on apoptotic blebs from *Leishmania major*-infected macrophages as a vaccine for cutaneous leishmaniasis. Apoptosis was induced in *Leishmania major*-infected J774A.1 cells in order to prepare apoptotic blebs. Test groups of BALB/c mice were immunized with these at doses of 1×10^6 , 5×10^6 or 1×10^7 blebs. An immunization control group received *Leishmania* lysate antigens. The results showed that as the number of apoptotic bodies increased, the lymphocyte proliferation index increased, and this was proportional to IFN- γ level in the test groups. Additionally, the difference of IFN- γ , IL-4, IFN- γ /IL-4 ratio, or total IgG ($p < 0.0001$) in all groups was statistically significant compared to the negative control group. The highest IFN- γ (514.0 ± 40.92 pg/mL) and IFN- γ /IL-4 ratio (2.94 ± 0.22) were observed in the group that received 1×10^7 apoptotic blebs. The highest levels of IL-4 (244.6 ± 38.8 pg/mL) and total IgG (5626 ± 377 μ g/mL) were observed in the immunization control group. Reflecting these data, no lesions were observed in any of the groups vaccinated with apoptotic blebs after 12 weeks. In summary, the use of apoptotic blebs from *Leishmania major*-infected macrophages is protective against the challenge with *Leishmania major* in this animal model.

Keywords

Apoptotic bleb; Cutaneous leishmaniasis; *Leishmania major*; Vaccination

49

50 **1. Introduction**

51 The Neglected Tropical Disease (NTD) leishmaniasis is endemic in 98 countries affecting
 52 approximately 12 million people per year with 350 million people living at risk of disease (World
 53 Health Organization). Causative *Leishmania* species are sand fly borne kinetoplastid parasitic
 54 protozoa [1] and infection can lead to a wide spectrum of clinical pathologies, from self-healing
 55 but scarring cutaneous leishmaniasis (CL) to fatal visceral disease (VL). Amongst other factors,
 56 this diversity of disease is dependent on the parasite species, host immunity, and genetic
 57 background [2]. Largely due to elimination efforts in south Asia, the global burden of VL has
 58 decreased substantially in the past decade. However, due to forced migration, the cases of CL
 59 have substantially increased in the same period [3]. Approximately, 75% of reported CL cases
 60 occur in 10 countries with an incidence rate of 0.7-1.3 million cases annually. However, the
 61 actual prevalence of this disease is estimated to be 6 to 10 times higher [4, 5].

62 Controlling reservoirs and vectors, providing diagnostics and treatment, and the emergence
 63 of drug-resistant species are serious challenges for the control of CL. These issues illustrate the
 64 need for new drugs for CL or effective vaccines [6-8]. There are three generations of vaccine
 65 used against leishmaniasis, 11 of which entered in clinical trials. These include Leishvaccine,
 66 ALM, Leishmune, CaniLeish, and GALM for first generation, LEISH-F1, LEISH-F2, LEISH-F3,
 67 Leish-Tec, and SMT^γ+NH^μ for second generation, and ChAd63-KH for third generation. Among
 68 these, Leishmune[®], CaniLeish[®], and Leish-Tec were approved to be used as vaccine in animal
 69 [9]. Therefore, there is still no appropriate vaccine available for human use.

70 In Iran in the 1970s and 1980s vaccination against CL widely used intradermal inoculation of
 71 live promastigotes of *L. major* ($2-3 \times 10^5$), leishmanization. However, this practice was

discontinued due to drawbacks such as ulcer development in a few susceptible individuals [10]. Immunity on leishmanization has been proposed to be due to vaccinated people harboring live parasites in their skin which release excretory/secretory antigens (ESAs), stimulating the host immune system and induce protection [11]. Accordingly, researchers have focused on *Leishmania* spp. ESAs as vaccine targets [12]. However, it is now known that the protective immunity against *L. major* is related to immune system memory, not the parasite presence in the skin tissue [13, 14]. In light of this, studies have focused on several types of vaccine, including live or live-attenuated parasites, the whole killed parasites, *Leishmania* spp. antigens and naked DNA-encoding parasite antigens. However, none so far have had the efficacy to be developed for use as a vaccine in humans [15, 16]. Against this backdrop, we focused on a new approach to vaccination against CL: apoptotic blebs from *Leishmania major*-infected macrophages.

Apoptotic blebs/bodies contain phosphatidylserine and phosphorylcholine on their surface, facilitating the clearance of these bodies by antigen presenting cells such as macrophages [17]. After phagocytosis of an apoptotic bleb, the antigen presenting cells process and present antigenic epitopes to the adaptive immune cells on class I and class II major histocompatibility complex (MHC-I and -II) molecule. However, there is preferential antigen presentation on MHC-I and therefore the cell-mediated immune response is stimulated, this is important for an effective CL vaccine [18-21]. Therefore, in this study we evaluated apoptotic blebs from *Leishmania major*-infected macrophages as a vaccine candidate for CL in a murine model.

2. Materials and Methods

2.1. *Leishmania* maintenance

Leishmania major (MRHO/IR/75/ER) was used in the present study. Promastigotes were cultured and maintained in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented by fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and penicillin-streptomycin solution (pen-strep; 100 IU/mL penicillin and 100 µg/mL streptomycin; Sigma-Aldrich, Missouri, USA). In order to reduce the proliferation of the parasite and to maintain it without passage for about 2 months, the amount of FBS was adjusted to 2% and the number of parasites to 10,000/mL. In addition, the culture medium containing the parasite was incubated at 24 °C for 24 hours and then kept at 4 °C for long-term maintenance. Daily monitoring of the growth of the parasite and the absence of fungal and bacterial contamination was undertaken [22].

2.2. Animal model

8-10 week-old female BALB/c mice (Pasteur Institute, Tehran, Iran), 20-25 gram, were utilized. The project underwent ethical review and was approved by the Ethics Committee of Mazandaran University of Medical Sciences (Code: IR.MAZUMS.REC.1397.315). The care and use of experimental animals complied with local animal welfare laws, guidelines, and policies.

2.3. Apoptosis induction in *Leishmania major*-infected macrophages

The cell line macrophages (J774A.1) were cultured in the same medium as used for the parasite culture but with 20% FBS as complete medium. 2×10^6 cells, counted using a Neubauer haemocytometer, were added to each 75 cm² cell culture flask (Corning, Oneonta, NY, USA), containing 10 mL of the complete medium. The flasks were incubated at 37 °C, 5% CO₂, and 95% humidity for 5-7 days (80% confluent, approximately 9.4×10^6 cells per flask) and 10 stationary phase promastigotes per macrophage (9.4×10^7) were added. The infection rate of

macrophage was calculated as 90.7% to 92.8%. After a further 24 hour incubation, apoptosis was induced in the infected cells using the Apoptosis Inducer Kit (Abcam, Cambridge, UK) according to the manufacturer's instruction. Afterwards, the suspensions obtained were filtered using a 5 μ m pore size membrane filter (Corning, Oneonta, NY, USA) and some of the solution was cultured and the parasite did not grow in the medium. The solution was centrifuged at 20,000 \times g for 15 minutes, the supernatants were discarded, and 1 mL of normal saline (0.85% w/v NaCl) was added to the precipitate. Following evaluation as below, the suspension of apoptotic blebs was stored at -20 °C until use.

2.4. Differentiation of apoptotic- from necrotic bodies by flow cytometry

The Annexin V-FITC Apoptosis Staining/Detection Kit (Abcam, Cambridge, UK) was used to differentiate apoptotic from necrotic bodies. The reaction mixtures were prepared according to manufacturer's instruction and read using as Coulter[®] Epics XL-MCL™ Flow Cytometer (Beckman Coulter, Fullerton, CA, USA). Briefly, four situations could occur: intact living cells which do not stain with both annexin V and PI; early apoptotic cells that stain with annexin V but do not stain with PI; late apoptotic cells that stain with both annexin V and PI; and necrotic cells that stain with PI but not annexin V.

2.5. Apoptotic blebs examination using scanning electron microscope (SEM)

The apoptotic bleb-containing solution were affixed to the surface of a metal stub, coated with gold through a SEM sputter coater (Bio-Rad, West Chester, PA, USA) and examined using Hitachi S-4300 SEM (Hitachi Science Systems, Tokyo, Japan) at accelerating voltages between 5 and 25 kV.

140

141 2.6. Preparation of *Leishmania* lysate antigens

142 As an immunization control, *Leishmania* lysate antigens (LLAs) were prepared by lysis of *L.*
 143 *major* promastigotes. 1×10^9 promastigotes in 5 mL of normal saline were lysed by bead-beating
 144 using 0.5 mm diameter glass beads (Sigma, Tokyo, Japan), 2 mL of beads were added to the
 145 suspension and this was vortexed vigorously for 5 minutes in 1 minute cycles with 10 seconds on
 146 ice between. The resulting lysate was passed through a 0.22 μ m pore size membrane filter
 147 (Corning, Oneonta, NY, USA), and the protein concentration was measured by the Bradford
 148 method [23]. The LLAs solution was stored at -20 °C until use.

149

150 2.7. Immunization of mice

151 55 BALB/c mice were randomly divided into 5 groups of 11, of which three test groups received
 152 1×10^6 , 5×10^6 or 1×10^7 apoptotic blebs, prepared above, in 0.5 mL of normal saline. One of
 153 the two remaining groups was considered as immunization control and received 100 μ g of LLAs,
 154 the remaining group served as the negative control and received 100 μ L of normal saline. The
 155 groups were subcutaneously inoculated twice at intervals of 14 days.

156

157 2.8. Cytokine and antibody assay

158 2 weeks after the second injection, approximately 300 μ L of blood was taken from the tail of the
 159 mice. The samples were centrifuged at 2000 \times g, 4 °C, for 10 minutes, the serum was separated
 160 and kept at -20 °C until use. Mouse IFN Gamma PicoKine™ ELISA Kit, Mouse IL-4 PicoKine
 161 ™ ELISA Kit, and Mouse Total IgG PicoKine ™ ELISA (BosterBio, Wuhan, China) were used
 162 to measure the levels of IFN- γ , IL-4 and total IgG respectively in the serum samples according to

the manufacturer's protocol. The reactions were analyzed using Bio-Rad Model 680 Microplate Reader (Bio-Rad, Hercules, CA, USA) and the value from each sample was calculated according to the plotted standard curve provided in the kits.

2.9. Lymphocyte proliferation assay

24 hours after blood sampling, 5 mice from each group were randomly selected and euthanized. Subsequently, 5 ml of normal saline was immediately injected into the peritoneal cavity of each mouse and the leukocytes containing peritoneal fluid were harvested. To isolate lymphocytes from peritoneal macrophages the cell suspension of each mouse was poured into separate 12.5 cm² cell culture flasks (Corning, Oneonta, NY, USA) and incubated at 37 °C, 5% CO₂, and 95% humidity for 3 hours. The supernatants, containing lymphocytes, were then harvested and centrifuged at 400 ×g, 4 °C for 10 min. The supernatants were discarded and 1 mL of phenol red free RPMI-1640 with pen-strep and 5% FBS was added to the precipitates. For each mouse 5 × 10⁵ lymphocytes, in 100 µL of media, were added into 9 wells of a 96-well microplate (BrandTech Scientific, Essex, CT, USA). 10 µg of LLAs was added to three wells as the immunization control and the mitogen PHA (2.5 µg/mL) was added to three as the control for normalization [24]. The remaining three wells were considered the negative control and nothing was added. The final volume of each well was adjusted to 150 µL using media. The plates were incubated at 37 °C, 5% CO₂, and 95% humidity for 48 hours. Subsequently, 20 µL of MTT stock solution (5 mg/mL; Gibco, Carlsbad, CA, USA) was added to each well and the volume adjusted to 200 µL using the medium (final concentration of MTT was 0.5 mg/mL). The plates were incubated as above for 4 hours and then centrifuged at 400 ×g for 10 minutes. The supernatants were discarded and 100 µL of acidic dimethyl sulfoxide (DMSO 1% of 1N HCl; Sigma-Aldrich,

Missouri, USA) was added to each well. The wells were rotated gently for 10 minutes using shaking incubator (Eppendorf, Hamburg, Germany), centrifuged at $10,000 \times g$ for 5 minutes and the supernatants were collected and analyzed using a scanning multiwell spectrophotometer (Biotek, Burlington, VT, USA) at the wavelength of 540 nm. The obtained optical densities (ODs) were analyzed according the following formula:

Firstly, for each mouse the stimulation index (SI) of lymphocytes was calculated for the treated lymphocytes with LLAs and PHA as follows:

$$\text{SI (LLA)}: \frac{\text{mean OD of three LLA wells} - \text{mean OD of three NC wells}}{\text{mean OD of three NC wells}}$$

$$\text{SI (PHA)}: \frac{\text{mean OD of three PHA wells} - \text{mean OD of three NC wells}}{\text{mean OD of three NC wells}}$$

NC is negative control

Then:

$$\Delta \text{SI} = \text{SI (LLA)} - \text{SI (PHA)}$$

The mean ΔSI was calculated for each group of 5 mice.

Finally, the proliferation index (PI) was calculated:

$$\text{PI} = 2^{(\Delta \text{SI of the treated group} - \Delta \text{SI of the NC group})}$$

NC is the negative control group (i.e. untreated mouse group); the treated group is each of the mice groups treated with apoptotic blebs or LLA.

PI indicates the potency of lymphocyte proliferation in response to injection of each treated group.

2.10. Infection challenge

The 6 mice which remained in each group were utilized in a challenge experiment [25]. 5×10^5 stationary phase promastigotes in a 10- μ L volume were inoculated intradermally 3-4 mm from the base of the tail of the mice. The mice were monitored weekly for 12 weeks for lesion development. The formula used in our study was that for ellipsoid lesions [26]:

$$LS = ([A/2 \times B/2 \times C/2] \times 4/3\pi)$$

Where LS is the lesion size in mm^3 ; “A” is depth of the lesion in mm; “B” and “C” respectively are the width and length of the lesion in mm.

2.11. Data analyses

Shapiro-Wilk statistical test was used to measure normal distribution of data. Data were analyzed using IBM SPSS v16 software (IBM Corp., Armonk, NY, USA) based on the two-tailed *t*-test and analysis of variance (ANOVA) statistical methods. In addition, *post hoc* comparison was performed between groups using the Tukey’s HSD test. The statistical differences were considered significant where *p* value < 0.05. The ELISA data were analyzed using GraphPad Prism v6 software (GraphPad, La Jolla, CA, USA).

3. Results

3.1. Quantity and quality of apoptotic blebs from *Leishmania major*-infected macrophages

The blebs were quantified using flow cytometry. 86.6% of the bodies identified were apoptotic, of which 30.1% were early apoptotic bodies and 56.5% were late apoptotic bodies. The total number of these bodies was 20,606,700 per mL (Figure 1).

The quality of the apoptotic bodies was established using SEM. The average size was 3.88 μm (95% CI 2.69-5.07), and the bodies were uniform and slightly oval (> 98%) in shape. All had an intact cell membrane (Figure 2).

3.2. Cytokine and antibody assay

IFN- γ , IL-4, and total IgG levels were assessed in the serum samples from the immunized mice. Statistical analyses, using ANOVA, showed that there was a significant difference for the levels of each of total IgG, IFN- γ and IL-4, as well as the IFN- γ /IL-4 ratio, among the groups of mice ($p < 0.0001$) (Table 1). Moreover, the pairwise comparison between the groups is shown in Table 2. In addition, the lowest levels of total IgG ($1522 \pm 327 \mu\text{g/mL}$), IFN- γ ($153.1 \pm 27.3 \text{ pg/mL}$), IL-4 ($135.3 \pm 28.5 \text{ pg/mL}$), and the lowest IFN- γ /IL-4 ratio (1.13 ± 0.07) were observed in the negative control group. The highest level of IFN- γ ($514.0 \pm 40.92 \text{ pg/mL}$) and IFN- γ /IL-4 ratio (2.94 ± 0.22) was observed in the group that received 1×10^7 apoptotic blebs (maximum dose). The highest levels of IL-4 ($244.6 \pm 38.8 \text{ pg/mL}$) and total IgG ($5626 \pm 377 \mu\text{g/mL}$) were observed in the immunization control group (Figure 3).

3.3. Lymphocyte proliferation assay

The proliferation index of the three groups that received apoptotic blebs and the immunization control LLA group, was calculated relative to the negative control group ($\Delta\text{SI} = -1.1$). The lowest proliferation index was observed for the group that received 1×10^6 apoptotic blebs (8.75) and the highest (24.25) was for the group that received 1×10^7 apoptotic blebs (Figure 4). The results of statistical analysis between the groups are shown in Figure 4.

3.4. Lesion size evaluation

No lesion development was observed in any of the groups that received apoptotic blebs. However, lesion development was observed in the mice from the negative and immunization control LLA groups. Maximum lesion size was $11.7 \pm 1.03 \text{ mm}^3$ in the negative control group and $5.89 \pm 0.19 \text{ mm}^3$ in the immunization control group at the 12th week. The mean \pm standard deviation of the lesion size in mm^3 in the mice of the negative and immunization control groups from week 1 to week 12 is shown in Figure 5. There was a statistically significant difference between the negative control group and each of the groups received apoptotic blebs ($p < 0.0001$). Moreover, a significant statistical difference was observed between the immunization control group and each of the groups received apoptotic blebs ($p < 0.001$).

4. Discussion

There are many challenges for the development of a CL vaccine, including the genetic diversity in human populations and in the species and strains of *Leishmania* parasite, the type of vaccine, dose of vaccine, and route of vaccine administration [27]. The purpose of the vaccination is the development of immunological memory, and both CD4^+ and CD8^+ T cells are important for the immunity against *L. major*. Therefore, it is clear that an effective vaccine should not only be safe and easily available but also should be capable of supporting the prolonged induction of CD4^+ and CD8^+ T lymphocytes [28].

The lymphocyte proliferation test is a non-specific test used to evaluate the lymphocyte response to specific antigens and is useful to evaluate the efficacy of a vaccine [29, 30]. In the reported study, it was observed that as the number of apoptotic bodies increased, the lymphocyte proliferation index increased and this was proportional to $\text{IFN-}\gamma$ measured in the test groups.

Previously, the lymphocyte proliferation response was used to assess three parasitic antigens as vaccines, the lowest proliferation index was observed in the LLAs injected group and this was consistent with the measured IFN- γ [31]. Therefore, lymphocyte proliferation is an indicator of the cellular immune response, especially IFN- γ production [32].

One component of a vaccine is usually an adjuvant to enhance specific immune responses and increase vaccine efficacy. *L. major* ribosomal protein (LRP) was evaluated as a vaccine alone or in combination with CpG oligodeoxynucleotides (CpG-ODN) as an adjuvant. Injection of the vaccine with the adjuvant (LRP+CpG) significantly increased the IFN- γ response in splenocyte supernatants of the mice whilst considerably decreasing the amount of IL-4 and IL-10, with IFN- γ /IL-10 ratio ~ 40 and IFN- γ /IL-4 ratio ~ 100 [33]. This IFN- γ /IL-4 ratio is much larger than IFN- γ /IL-4 ratio of the group that received 1×10^6 the apoptotic blebs (95% CI 1.37-1.45) in the reported study. Iborra, Parody [33] showed that the immune responses prevented the formation of lesions in the challenged BALB/c mice; however, some swelling was observed in the LRP+CpG vaccinated mice and the few parasites were found in the tissue. In the reported study, no lesions were observed in the challenged mice received apoptotic blebs, precluding the measurement of parasite burden.

Few adjuvants are acceptable for human use, and there are many limitations to introducing a new one [34]. Interestingly, apoptotic blebs can be both a vaccine and an adjuvant in that foreign antigen containing apoptotic bodies act as natural adjuvants that stimulate a specific cellular immune response mediated by CD8⁺ T cell, enhancing the vaccine efficacy and providing stable immunity [35-37]. The results of the reported study support this approach, however, to evaluate the role of host factors in the induction of immunity observed in this study, future work will need

to analyze the effects of apoptotic blebs from uninfected macrophages on the induction of an immune response.

LLAs-loaded liposomes have also resulted in an improved, although non-protective, immune response, which increased IFN- γ and reduced IL-4 compared to LLAs alone [38]. The highest amount of total IgG was quantified in the LLAs alone group [38]. Similarly, in the reported study, the highest level of total IgG was observed in the LLAs group, consistent with the high level of IL-4. Furthermore, LLAs have been shown to induce a higher IgG response than recombinant *Leishmania* homolog of receptors for activated C-kinase (rLACK) antigens in BALB/c mice [39]. An increase in the amount of IFN- γ level and a decrease in IL-10 level were also observed in splenocyte supernatants of the rLACK group compared to the LLAs group, with an IFN- γ /IL-10 ratio of 55 for the rLACK group compared to 15.3 for LLAs. This indicated more potent cellular immune response to rLACK, however, this response was not protective against *L. major* challenge [39]. Similarly, in the reported study, IgG and IL-4 were higher in the LLAs group than in the test groups (i.e. the received apoptotic blebs). However the IFN- γ /IL-4 ratio for rLACK (~ 329) was very much larger than in the apoptotic blebs test groups (1.41-2.94 depending on dose), despite protective immunity being observed in the latter but not the former.

The quantity of IFN- γ produced on vaccination is considered to be the most important factor in the development of a protective immune response against CL [40, 41]. Interestingly, despite the lack of lesion growth in the groups that received apoptotic blebs, IFN- γ response in the mouse sera was much lower than that obtained in other studies that found no protection against challenge [38, 39]. To evaluate the effect of IFN- γ on preventing lesion development, IL-12 together with *L. braziliensis* was inoculated into a murine model and a significant increase in IFN- γ was observed. However, this did not prevent *L. brasiliensis* lesion development [42].

Consequently, it can be concluded that factors other than the quantity of IFN- γ and IL-4, and the IFN- γ /IL-4 ratio, are central to the development of a protective immune response for CL.

Clearly our understanding of IFN- γ and IL-4 function in CL is incomplete, IL-4 and IL-4RA knockout mice are rendered susceptible to challenge with *L. major* [43]. Furthermore, it is expected that during the lesion healing, IL-4 will be reduced and IFN- γ increased, however the expression level IL-4 in the margin of the late lesions was found be to higher than IFN- γ [44].

5. Conclusions

In the presented study, apoptosis was induced in *Leishmania*-infected macrophages and the apoptotic-blebs collected. These bodies will contain fragments of the parasite and together can function as analogous to a whole *Leishmania*. Whilst the mechanism of inducing protective immune response has not been evaluated for the apoptotic-blebs from *Leishmania major*-infected macrophages in the current study, it is likely that these ‘fragments’ constitute a complex antigenic picture necessary for immunity. The results showed that vaccination with even the lowest number (1×10^6) tested of these apoptotic-blebs, is protective against *L. major* challenge. In addition, we propose that a factor other than the quantity of IFN- γ and the IFN- γ /IL-4 ratio is likely to play a role in providing this protection, this will be the focus of future studies.

Conflicts of interest

The authors declare that there is no conflict of interest.

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Table 1. The results of ANOVA statistical test for ELISA data in the examined groups

Groups	IFN- γ (pg/mL)				IL-4 (pg/mL)				IFN- γ /IL-4 ratio				IgG (μ g/mL)			
	Mean \pm SD	95% CI	F value	p value	Mean \pm SD	95% CI	F value	p value	Mean \pm SD	95% CI	F value	p value	Mean \pm SD	95% CI	F value	p value
AB 1 ^a	261.9 \pm 37.31	236-287	134.4	< 0.0001	184.2 \pm 24	168-200.3	19.79	< 0.0001	1.416 \pm 0.06	1.37-1.45	469.7	< 0.0001	3068 \pm 373	2817-3319	168.3	< 0.0001
AB 2 ^b	270.0 \pm 42.37	241.5-298.5			175.8 \pm 25.7	158.6-193.1			1.505 \pm 0.04	1.47-1.53			3778 \pm 423	3494-4062		
AB 3 ^c	514.0 \pm 40.92	486.5-541.5			175 \pm 27.3	156.7-193.3			2.94 \pm 0.22	2.79-3.1			3984 \pm 397	3717-4251		
PC ^d	279.6 \pm 39.48	253.1-306.2			244.6 \pm 38.8	218.5-270.7			1.14 \pm 0.06	1.1-1.18			5626 \pm 377	5373-5880		
NC ^e	153.1 \pm 27.3	134.8-171.4			135.3 \pm 28.5	116.1-154.4			1.13 \pm 0.07	1.08-1.18			1522 \pm 327	1302-1741		

^a AB 1: the group that received 1×10^6 apoptotic blebs from *Leishmania major*-infected macrophages^b AB 2: the group that received 5×10^6 apoptotic blebs from *Leishmania major*-infected macrophages^c AB 3: the group that received 1×10^7 apoptotic blebs from *Leishmania major*-infected macrophages^d PC: the immunization control group that received *Leishmania* lysate antigens^e NC: the negative control group

466 **Table 2.** Pairwise comparison using *post hoc* Tukey's HSD statistical test for ELISA data^a

IFN- γ (pg/mL)	AB 1	AB 2	AB 3	PC	NC	IL-4 (pg/mL)	AB 1	AB 2	AB 3	PC	NC
AB 1 ^b	0	8.09	252.1****	17.73	108.8****	AB 1	0	8.36	9.18	60.45***	48.91**
AB 2 ^c		0	244.0****	9.63	116.9****	AB 2		0	0.81	68.82****	40.5468
AB 3 ^d			0	234.4****	360.9****	AB 3			0	69.64****	39.73*
PC ^e				0	126.5****	PC				0	109.4****
NC ^f					0	NC					0

IFN- γ /IL-4 ratio	AB 1	AB 2	AB 3	PC	NC	IgG (μ g/mL)	AB 1	AB 2	AB 3	PC	NC
AB 1	0	0.08	1.53****	0.27****	0.28****	AB 1	0	709.7***	916.2****	2558****	1546****
AB 2		0	1.44****	0.36****	0.37****	AB 2		0	206.5	1849****	225471
AB 3			0	1.8****	1.81****	AB 3			0	1642****	2463****
PC				0	0.007	PC				0	4105****
NC					0	NC					0

473 ^a Mean difference shown

474 ^b AB 1: the group that received 1×10^6 apoptotic blebs from *Leishmania major*-infected macrophages

475 ^c AB 2: the group that received 5×10^6 apoptotic blebs from *Leishmania major*-infected macrophages

476 ^d AB 3: the group that received 1×10^7 apoptotic blebs from *Leishmania major*-infected macrophages

477 ^e PC: the immunization control group that received *Leishmania* lysate antigens

478 ^f NC: the negative control group

479 * $p < 0.05$

480 ** $p < 0.01$

481 *** $p < 0.001$

482 **** $p < 0.0001$

Figure legends:

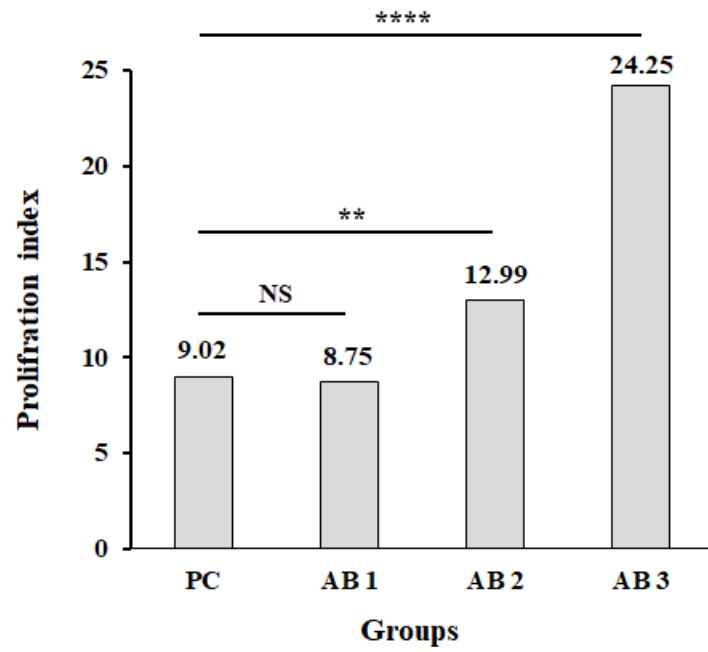
Figure 1. Detection of apoptotic blebs using flow cytometry. Q1: necrotic bodies; Q2: late apoptotic; Q3: live cells; Q4: early apoptotic. The scales represent relative intensity of fluorescence for each of the two fluorophores. The sample was diluted to one-sixtieth

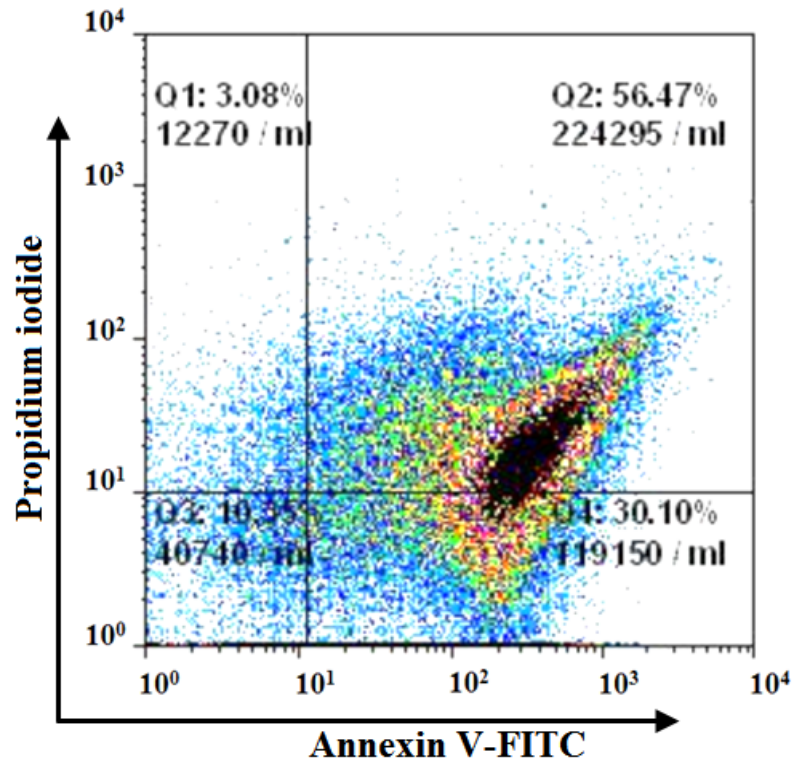
Figure 2. Detection of apoptotic blebs using scanning electron microscope (SEM)

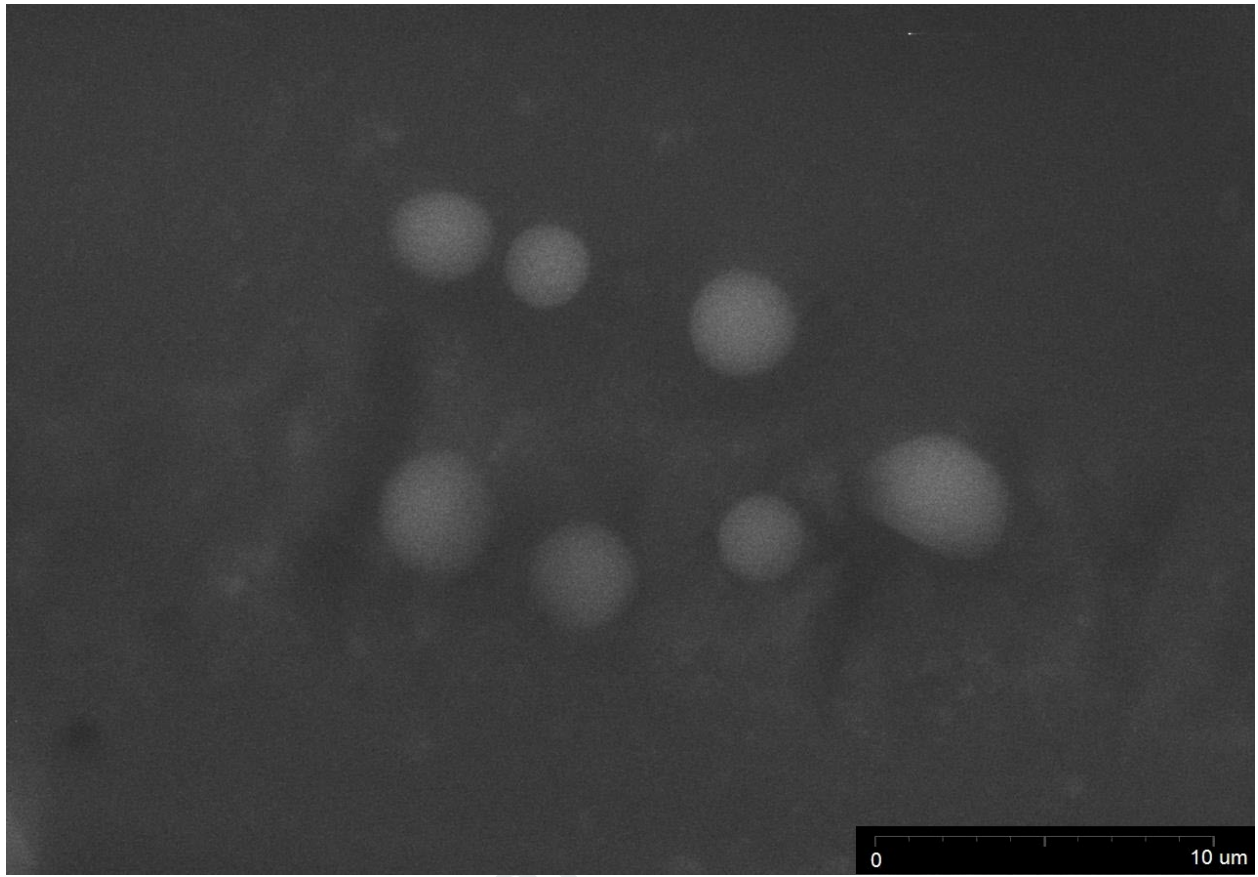
Figure 3. The amount of IFN- γ , IL-4, IFN- γ /IL-4 ratio, and total IgG in the serum samples of the examined mice. AB 1: the group that received 1×10^6 apoptotic blebs from *Leishmania major*-infected macrophages; AB 2: the group that received 5×10^6 apoptotic blebs from *Leishmania major*-infected macrophages; AB 3: the group that received 1×10^7 apoptotic blebs from *Leishmania major*-infected macrophages; PC: the immunization control group that received *Leishmania* lysate antigens; NC: the negative control group

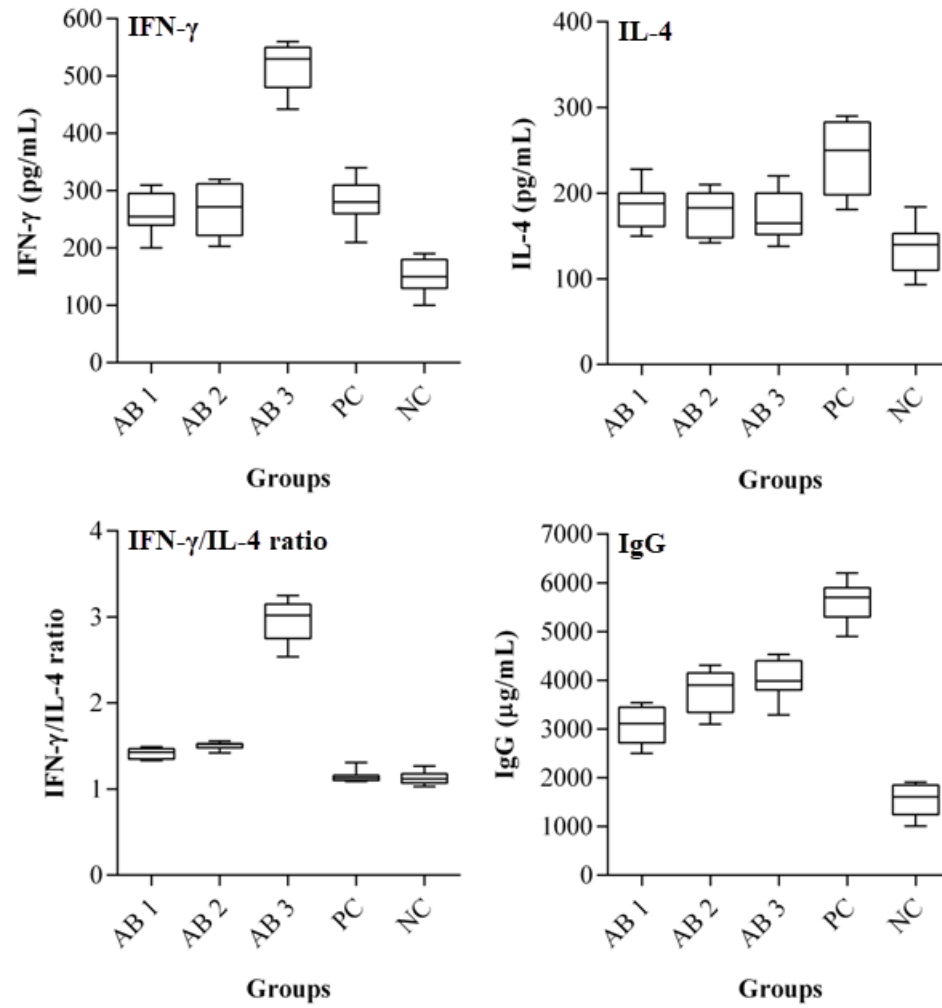
Figure 4. Proliferation index of lymphocytes in the treated groups relative to the negative control group. AB 1: the group that received 1×10^6 apoptotic blebs from *Leishmania major*-infected macrophages; AB 2: the group that received 5×10^6 apoptotic blebs from *Leishmania major*-infected macrophages; AB 3: the group that received 1×10^7 apoptotic blebs from *Leishmania major*-infected macrophages; PC: the immunization control group that received *Leishmania* lysate antigens. ** $p < 0.01$; **** $p < 0.0001$; NS = not significant

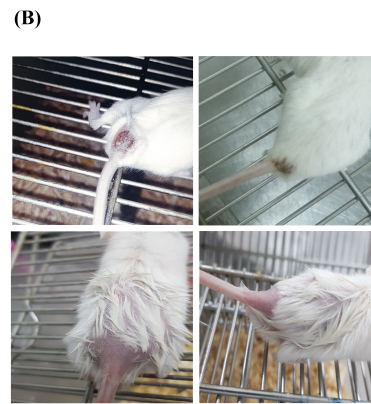
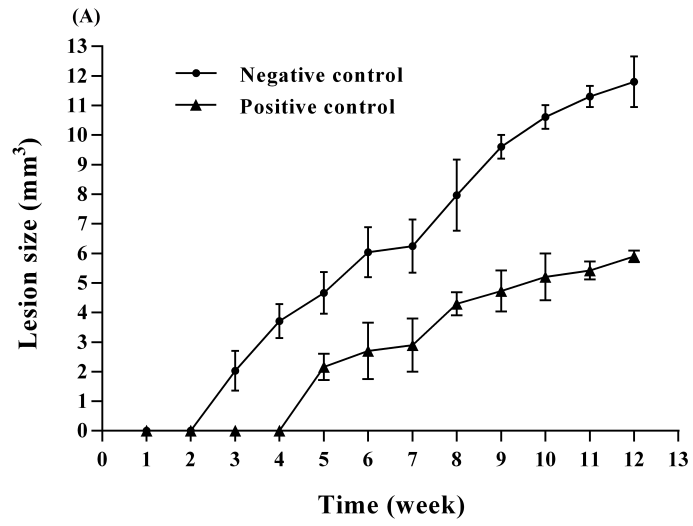
Figure 5. The process of lesion formation from the beginning to the end of the examination. (A) Lesion size in the negative and immunization control groups. (B) Top-left picture shows the lesion in the negative control group. Top-right picture shows the lesion in the immunization control group. The two pictures below show that the mice receiving apoptotic bodies did not develop lesion. Immunization control = LLAs group











Highlights:

- No lesions were observed in the groups vaccinated with apoptotic blebs from *Leishmania major*-infected macrophages
- Apoptotic blebs contain a wide variety of the parasite antigens and may stimulate immune responses similar to whole killed parasite
- The highest IFN- γ and IFN- γ /IL-4 ratio were observed in the group that received 1×10^7 apoptotic blebs
- The immunization with apoptotic blebs protected mice from cutaneous swelling against *Leishmania major* challenge

Author Statement:

Roghiyeh Faridnia: *Leishmania* Maintenance, Apoptosis Induction, Preparation of *Leishmania* Lysate Antigens, Immunization of Mice. **Hamed Kalani:** Flow Cytometry, Scanning Electron Microscope, Lymphocyte Proliferation Assay, Design of the Study, Drafting the Manuscript. **Hajar Ziaei Hezarjaribi:** Immunization of Mice. **Paul W. Denny:** Writing-Reviewing and Editing. **Alireza Rafie:** Cytokine and Antibody Assay. **Mahdi Fakhar:** Infection Challenge, Data analysis, Writing and Editing. **Stela Virgilio:** Reviewing and Editing.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: